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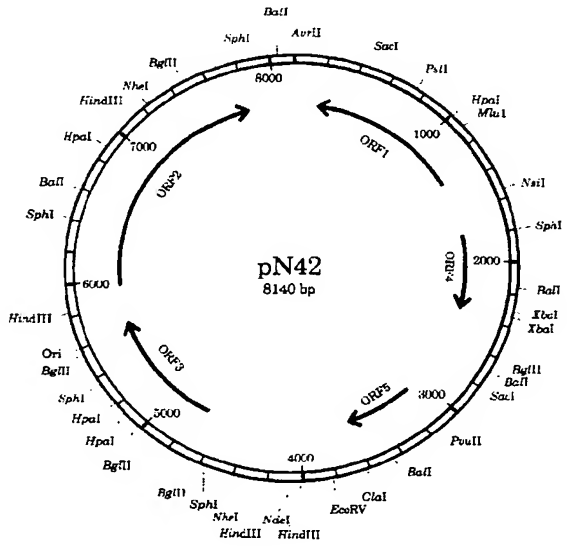
## (54) Plasmid derived from Lactobacillus delbrueckii sp.

(57) The present invention concerns a plasmid derived from Lactobacillus delbrueckii sp. comprising at least the restriction map of the Figure 1 or portion(s) thereof ; the recombinant vector comprising the said plasmid, at least one DNA sequence capable of replication into E. coli and/or Lc. lactis and at least one marker.

The present invention concerns also the microorganism transformed by the said plasmid and/or by the said recombinant vector.

FIG.1

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## Field of the invention

The present invention concerns a new plasmid derived from Lactobacillus delbrueckii sp., a recombinant vector comprising said plasmid, the microorganism transformed by said plasmid and/or vector and the use of the plasmid and/or the vector for the transformation of microorganisms.

## Background of the invention and state of the art

A successful biological transformation of an organism must satisfy the following three criteria:

1. Transforming DNA must enter the organism by physical or chemical means such as electrotransformation, treatment with inorganic ions, protoplast fusion, etc.
2. Transformants must be selected with the help of one or more markers from the non transformed cells in the population for instance by antibiotic resistance genes linked to the transforming DNA. This is best satisfied by either the isolation of a resistance gene against an antibiotic from the target host in question, or by the engineering of a known resistance gene with expression sequences (promoter and terminator) compatible with the target host.
3. Transforming DNA must be replicated (either autonomously or as part of the host genome). This is best satisfied by the isolation of replicating plasmids from the host to be transformed and to subsequently construct vectors able to replicate in a microorganism such as Escherichia coli (E. coli) or Lactococcus lactis (Lc. lactis) and in a specific target organism such as Lactobacillus delbrueckii subsp. bulgaricus (L. bulgaricus).

The international patent application W092/14825 describes a plasmid pBULI having a length of about 7.9 kb and its derivative isolated from Lactobacillus delbrueckii subsp. bulgaricus M-878 strain.

The restriction map of this plasmid is characterized by the absence of restriction sites for BamHI, EcoRI, KpnI and PstI enzymes.

This plasmid is used as a vector for breeding various microorganisms such as lactic acid bacteria and the derivative of this plasmid is used as a shuttle vector (lactic acid bacterium - Escherichia coli).

Other shuttle vectors are described in the documents Canadian Journal of Microbiology (vol. 38 (1992) pp 69-74), ACTA MICROBIOLOGICA BULGARICA (vol. 27 (1991) 99 3-8) and in the Japanese Patent Application JP-A-4.218.381.

## Aims of the invention

The present invention aims to provide a new plasmid derived from Lactobacillus delbrueckii sp. which can be used to transform specific microorganisms specially Lactobacillus bulgaricus.

Another aim of the invention is to obtain a recombinant vector comprising the said plasmid and which can replicate in E. coli and Lc. lactis and transform specific microorganisms, specially Lactobacillus bulgaricus.

## Disclosure of the invention

The present invention concerns a new plasmid derived from Lactobacillus delbrueckii sp. comprising at least the restriction map of the Figure 1 or portion(s) thereof.

Preferably said portion is a sufficient amount of the restriction map of the Figure 1, so as to provide all the plasmid encoded TRANS and CIS elements necessary for replication of the plasmid in Lactobacillus bulgaricus.

The plasmid according to the invention comprises at least the DNA sequence SEQ ID N° 1 and/or its complementary strand, or portion(s) thereof.

Preferably, said portion is a sufficient amount of the DNA sequence SEQ ID N° 1 and/or its complementary strand so as to provide all the plasmid encoded TRANS and CIS elements necessary for replication of the plasmid in Lactobacillus bulgaricus.

Furthermore, the present invention concerns a recombinant vector comprising the plasmid according to the invention, at least one DNA sequence capable of replication in E. coli and/or Lc. lactis and at least one marker.

The DNA sequence capable of replication in E. coli and/or Lc. lactis is constituted for instance by a specific plasmid, such as pDP193, which allows the recombinant vector to be freely cultured in either E. coli or Lc. lactis for molecular manipulations.

The marker comprised in the recombinant vector according to the invention, is a DNA fragment used as a reference for analytical purposes (i.e. a gene with known phenotype and mapped position) and/or a foreign

DNA fragment which is expressed in the microorganism transformed by the vector according to the invention.

This DNA fragment may be used also for the transformation of microorganisms in order to obtain for instance:

- resistant strains to phages,
- ropy strains (improved texturing properties),
- probiotic strains,
- strains producing new or improved enzymes (lipases, deshydrogenases,...), aroma or flavor compounds,...

The present invention concerns also the microorganism, preferably Lactobacillus bulgaricus, transformed by the plasmid and/or by the recombinant vector according to the invention.

Finally, the present invention concerns the use of the plasmid and/or the vector according to the invention for the transformation of microorganisms.

## Brief description of the drawings

The Figure 1 represents the restriction map of the Lactobacillus delbrueckii sp. plasmid pN42 according to the invention.

The Figure 2 represents the construction of the plasmid pN42-Sub CB from the pJDC9 plasmid and pN42 plasmid.

The Figure 3 represents the construction of pN42-Sub CE from the pJDC9 plasmid and pN42 plasmid.

The Figure 4 represents the construction of pN42-Sub W and pN42-Sub X from the pUC19 plasmid and pN42 plasmid.

The Figure 5 represents the construction of chloramphenicol transacetylase gene of pDP352.

The Figure 6 represents the construction of the pDP193 plasmid.

The Figure 7 represents the construction of pDP359 plasmid.

## Description of a preferred embodiment of the invention

The construction of pDP359, a E. coli/Lc. lactis-L. delbrueckii sp. shuttle vector according to the invention is characterized by the following features.

Firstly the incorporation of pDP193 allows the plasmid to be freely cultured in either E. coli or Lc. lactis for molecular manipulation, such as the addition of genes to be expressed in L. bulgaricus. Secondly the inclusion of a bona fide L. delbrueckii sp. plasmid in its entirety ensures that pDP359 contains all the sequences required for the replication of pN42 and hence must replicate in L. bulgaricus in the same fashion as pN42 in its host N42. Thirdly the inclusion of the chloramphenicol resistance gene engineered in pDP352 ensures a means to select for transformants in L. bulgaricus.

Analysis of over fifty L. delbrueckii sp. strains from the Nestle culture collection identified one, N42, that contains an extra-chromosomal replication plasmid. This is designated pN42 (its restriction map is shown in the figure 1)and chosen for analysis as it must contain all of the plasmid encoded TRANS and CIS elements necessary for its replication in L. bulgaricus. The integrity of N42 as a L. delbrueckii sp. is ascertained by API tests and molecular characterization of hybridization with the L. delbrueckii specific probe (Delley M., Mollet B., and Hottinger H., 1990, DNA probe for Lactobacillus delbrueckii, Appl. Environ. Microbiol, 56:1967-1970).

pN42 plasmid DNA is isolated by cesium chlorideethidium bromide buoyant density gradients for restriction mapping and sub cloning. Plasmid pN42 is cloned in its entirety into the E. coli vector pJDC9 (J.-D. Chen and D.A. Morrisson 1987, Cloning of Streptococcus pneumoniae DNA Fragments in Escherichia coli Requires Vector Protected by Strong Transciptional Terminators, Gene 55, 179-187) at several identified unique restriction sites PstI (pN42-Sub CB), AvrII (pN42-Sub CE) or into the pUC/pK plasmids for DNA sequence analysis.

pN42 plasmid DNA is digested with the restriction enzyme PstI, mixed with PstI digested and dephosphorylated pJDC9 vector, ligated and transformed into E. coli. Colonies are analyzed by restriction enzyme digestions and a positive clone designated pN42-Sub CB (figure 2).

pN42 plasmid DNA is digested with the restriction enzyme AvrII, mixed with XbaI digested and dephosphorylated pJDC9 vector, ligated and transformed into E. coli. Colonies are analyzed by restriction enzyme digestions and a positive clone designated pN42-Sub CE (figure 3).

Plasmid pN42-Sub CB is digested with the restriction enzymes EcoRV and PstI, the DNA fragments separated on an agarose gel and the 3.1 kb and 5.1 kb fragments purified. These two fragments are mixed with PstI and SmaI digested and dephosphorylated pUC19 vector, ligated and transformed into E. coli. Colonies are analyzed by restriction enzyme digestions and the positive clones designated PN42-Sub W and pN42-Sub X (for the 5.1 kb and 3.1 kb fragments respectively) (figure 4).

The complete DNA sequence of pN42 is determined from subclones from synthetic oligonucleotide primers on both strands by the dideoxy chain termination reactions using the T7sequencingo® kit of Pharmacia and $^{35}$SdATP. pN42 consists of a circular double stranded plasmid of 8140 base pairs with at least five open reading frames (designated ORF1 to ORF5) of 50 amino acids or more as identified by the computer program "Frames" from the GCG suite (Computer software is from Genetics Computer Group Inc. (GCG), Devereux J., Haeberli P. and Smithies O. (1984), A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12: 387-395). The GCG program "Repeat" identified a three times twenty-one base pair direct repeat which is the potential origin of replication. The restriction map of pN42 is shown in Figure 1 and the complete DNA sequence in sequence listing 1 (SEQ ID N° 1).

The DNA sequence analysis of pN42 allows the definition of structural features that may be important for the replication of the plasmid in L. delbrueckii sp. and the construction of shuttle vectors that include all these features intact (the introduction of genes may be obtained by cloning pN42 at the following restriction sites Avr II, NsiI, SphI, Nb plasmid DNA isolated from Lactobacillus delbueckii sp. digested at only one of the five SphI sites I.E. at bp 7882).

This ensures that the said shuttle vector must replicate when transformed into L. bulgaricus.

It is judged probable that antibiotic resistance conferred by a defined resistance gene may be transferred to any other organism if it contains the appropriate translation/transcriptional control signals. Therefore the defined gram positive chloramphenicol resistance gene (chloramphenicol acetyltransferase, CAT originally from Staphylococcus aureus) is been taken from the broad host range plasmid pNZ12 (W.M. de Vos, 1987, Gene Cloning and Expression in Lactic Streptococci, FEMS Microbiol. Reviews, 46, 281-295) and used to engineer the bona fide L. bulgaricus promoter from the lacS-Z operon (P. Leong-Morgenthaler, M.C. Zwahlen and H. Hottinger, 1991, Lactose Metabolism in Lactobacillus bulgaricus: Analysis of the Primary Structure and Expression of the Genes Involved, J. Bacteriol., 173, 1951-1957). This is followed with a gram positive stem-loop terminator from the lactose-galactose operon of Lc. lactis strain NCDO2054. The complete construction is shown in Figure 5.

The plasmid pKN19 is the E. coli cloning vector pK 19 (R.D. Pridmore, 1987, New and Versatile Cloning Vectors with Kanamycin-Resistance, Gene, 56, 309-312) where the unique BspHI restriction site in a non essential region is destroyed by restriction enzyme digestion and the four base overhang repared with Klenow enzyme and the four nucleotides according to Maniatis et al. (T. Maniatis, E.F. Fritch and J. Sambrook, Molecular cloning a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982). The chloramphenicol resistance gene from pNZ12 is extracted by PCR amplification (Saiki R.K., Gelfand D.H., Stoffel S., Scharf S.J., Higuchi R., Horn G.T., Mullis K.B., and Ehrlich H.A., 1988, Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 239: 487-491; Saiki R.K., Scharf S., Faloona F., Mullis K.B., Horn G.T., Ehrlich H.A. and Arnheim N., 1985, Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, Science 230: 1350-1354) using the mutagenic primers A (5'-AGGAGGATCCTCTCATGAACTTTAATAAAATTG) that introduced a BspHI restriction site overlapping the ATG initiation codon of the CAT gene, plus primer B (5'-TACAGTATCGATTATCTCATATTATA) that introduces a ClaI restriction site 9 bp down stream of the CAT gene. The PCR amplification is performed on 50 ng of BglII digested pNZ12 DNA with 0.3 µM each of oligonucleotides C plus D, 200 µM of the four nucleotides and PCR cycling at 94°C for 0.5 minutes, 50°C for 0.5 minutes, 72°C for 0.5 minutes for a total of 30 cycles.

The product is digested with the restriction enzymes ClaI plus BamHI and the 660 bp fragment purified from an agarose gel and cloned into the E. coli vector pBS KS+® (Stratagene Corp.) also digested with ClaI, BamHI and dephosphorylated. The ligated fragments are transformed into E. coli and plated onto LB plates supplemented with ampicillin, 5-bromo-4-chloro-3-indolyl-(3-D-galactopyranoside) (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG). Clones are screened by restriction enzyme digestions, a positive clone chosen and designated clone A; both chloramphenicol and ampicillin resistant. Clone A is digested with restriction enzymes MfeI, StuI and dephosphorylated. This fragment is replaced by the equivalent CAT MfeI-StuI fragment from pNZ12. This is to eliminate any PCR induced mutations within the CAT gene, giving Clone B. (This step is not shown in Figure 5).

Clone B is digested with the restriction enzymes BamHI plus ClaI and the 660 bp fragment purified from an agarose gel. pKN19/galT-term is pKN19 containing the Lc. lactis NCDO2054 lactose-galactose operon terminator as an SpeI-SacI restriction fragment, with its internal BspHI restriction site destroyed as described above. pKN19/galT-term is digested with the restriction enzymes SfuI plus SacI (both sites natural to the fragment) and the 190 bp fragment purified from an agarose gel. These two fragments are mixed together with the vector pKN19 digested with the restriction enzymes SacI, BamHI plus dephosphorylated, ligated together and transformed into E. coli. Clones are screened by restriction enzyme digestions, a positive clone chosen and designated clone C.

The published L. bulgaricus lacS promoter is used to design two mutagenic oligonucleotides, C (5'-ATTGGAAGAATTCACCAACGCTTTTCATTTC) which introduces an EcoRI restriction site 240 bp upstream of the ATG initiation codon and oligonucleotide D (5'-GGTGGTGACGAAGACGATA) which primes 110 bp down stream of the ATG of the lacS gene which naturally contains a BspHI restriction site overlapping the start codon. The PCR amplification is performed on 100 ng of genomic L. delbrueckii sp. DNA with 0.3 µM each of oligonucleotides C plus D, 200 µM of the four nucleotides and PCR cycling at 94°C for 0.5 minutes, 50°C for 0.5 minutes, 72°C for 0.5 minutes and a total of 30 cycles. The PCR product is digested with the restriction enzymes EcoRI plus BspHI and the 250 bp fragment purified from an agarose gel. Clone D is digested with the restriction enzymes BspHI plus SacI and the 780 bp fragment purified from an agarose gel. These two fragments are ligated together into EcoRI, SacI plus dephosphorylated pKN19 vector, transformed into E. coli, and plated onto LB plates supplemented with kanamycin. Clones are screened by restriction enzyme digestions, a positive clone chosen and designated pDP352 the complete DNA sequence of which is given in sequence listing 2 (SEQ ID No. 2).

The chloramphenicol resistance gene constructed in pDP352 is transcribed from a bona fide L. bulgaricus promoter that is constitutively expressed in this host. This includes the natural promoter elements of -35, -10 regions and the ribosome binding site at exactly the same relative position to the ATG of the chloramphenicol resistance gene as to the original ATG of the lacS gene. This ensures that the chloramphenicol resistance gene will be correctly transcribed and translation initiated at the correct position and that the resistance gene will work.

The E. coli-Lc. lactis shuttle vector pDP193 is constructed from the E. coli vector pUC18 (R.D. Pridmore, 1987, New and Versatile Cloning Vectors with Kanamycin-Resistance, Gene, 56, 309-312) plus the plasmid pVA749 (F.L. Macrina, J.A. Tobian, K.R. Jones and R.P. Evans, Molecular cloning in the Streptococci, in A. Hallaender, R. DeMoss, S. Kaplan, S. Konisky, D. Savage and R. Wolve (Eds.), Genetic engineering of microorganisms for chemicals, Plenum, New York, 1982, pp. 195-210). pVA749 is extracted from the chimeric plasmid pVA838 (F.L. Macrina, J.A. Tobian, K.R. Jones, R.P. Evans and D.B. Clewell, 1982, A Cloning Vector able to Replicate in Escherichia coli and Streptococcus sanguis, Gene, 19, 345-353) as a HindIII restriction fragment and cloned into the HindIII site of pUC18. The second HindIII site opposite to the pUC cloning array is removed by Klenow enzyme end repair. pVA749 itself consists of a gram positive plasmid origin of replication from Streptococcus faecalis (capable of replication in Lc. lactis) and the erythromycin resistance gene from pAMβ1. The construction of pDP193 is depicted in Figure 6.

Plasmid pVA838 is digested with the restriction enzyme HindIII, the fragments separated on an agarose gel and the 5.2 kb pVA749 fragment purified. Vector pUC18 is digested with the restriction enzyme HindIII, dephosphorylated, mixed with the pVA749 fragment, ligated and transformed into E. coli. Colonies are analyzed by restriction enzyme digestions and a positive clone designated Clone D. Clone D is digested with the restriction enzyme HindIII in the presence of 50 µg/ml ethidium bromide (M. Osterlund, H. Luthman, S.V. Nilsson and G. Magnusson (1982), Ethidium-bromide-inhibited restriction endonucleases cleave one strand of circular DNA, Gene 20, 121-125), the fragments separated on an agarose gel and the linear 7.9 kb fragment purified. The four base overhang generated by HindIII in the linear Clone D is filled in with Klenow enzyme in the presence of four nucleotides according to Maniatis et al. (T. Maniatis, E.F. Fritch and J. Sambrook, Molecular cloning a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), ligated and transformed into E. coli. Colonies are analyzed by restriction enzyme digestions and a positive clone designated pDP193.

Plasmid pDP193 is digested with the restriction enzymes SacI plus EcoRI and dephosphorylated. pDP352 is digested with the restriction enzymes SacI plus EcoRI and the 1100 bp CAT gene purified from an agarose gel. These two are mixed together, ligated and electrotransformed into the Lc. lactis plasmid free strain LM0230. Positive colonies are identified as erythromycin plus chloramphenicol resistant and confirmed by restriction enzyme digestions. A positive clone is chosen and designated pDP193-CAT 352.

pDP193-CAT 352 is digested with the restriction enzymes SseI plus BamHI and dephosphorylated. Plasmid pN42-Sub CE is digested with the restriction enzymes SseI plus BamHI (both sites from the linker) and the 9.3 kb fragment purified from an agarose gel. These two fragments are mixed, ligated and electrotransformed into Lc. lactis strain LM0230. Clones are screened by restriction enzyme digestions, a positive clone chosen and designated pDP359 as shown in figure 7.

The vector pDP359 satisfies the requirements for a shuttle vector for L. bulgaricus that must work in this host. It includes a complete bona fide replicating plasmid isolated and characterized from L. delbrueckii sp. plus a chloramphenicol resistance gene that is transcribed from a native L. bulgaricus promoter. These considerations ensure that the said plasmid pDP359 which replicate when introduced into L. bulgaricus.

# SEQUENCES LIST

Information for sequence ID No 1.

(i) Sequence characteristics:

(A) Length: 8140 base pairs
(B) Type: Nucleic acid
(C) Strandedness: Double
(D) Topology: Circular

(ii) Molecule type: DNA (plasmid)

(xi) Feature:
(vi) Origional source: Lactobacillus bulgaricus Strain N2.
(A) Name/key: Plasmid pN42
(B) Location: 1..8140

(XI) feature:
(A) Name/Key: Origin of replication.
(B) Location: 5694..5758.

(XI) feature:
(A) Name/Key: ORF1.
(B) Location: 1344..169.

(XI) feature:
(A) Name/Key: ORF2.
(B) Location: 5965..7806.

(XI) feature:
(A) Name/Key: ORF3.
(B) Location: 4718..5668.

(XI) feature:
(A) Name/Key: ORF4.
(B) Location: 3116..3637.

(XI) feature:
(A) Name/Key: ORF5.
(B) Location: 1779..2360.

CCTAGGCTTG AAATTGACGC ATAGGCGCAA AGGGAGCGGG CGACAGGGGG TAAAGCACGA 60
TAAATTCGTT TTTTACAGAC GTTCAGTCCA TGTTGTCATA TTTGTACTCC CGTTTTTAGG 120
GCTGTTTTAA AAGTATTTTT AGCGGCGATT TGTTAATTAT AGCCCCTATA CAAACATCTT 180
TTGTAAAAAG CCTTTTTTCT GTTCTTTCAA CAAATCTAAC TTACGTTGAT GAAGAGCGAT 240
AGTGTCATCT AGCTGTTTTA AAAATGAGCC TATTTTTTTT TGTTCTTCCT GACTAGGTTT 300
ATAGATTTTA AATGATGAAA ATTTAGAAAT CCAATGACGT TCATGACTTT GAGGTACATA 360
TTTTATATTC TTCAATGTAT TAAACATAAA ATAGAAATTG TCAGAATTAT CATTCAAACT 420
AAGTAATTTC ATTGCGGAGC TCTTAATTTT AAAAGGGAAA TCTACATAAT GAGAGTCAGT 480
TGTAAAATCA TCAAATATAA CAACTGGATT TTCTACGGTA GCATTTTTAA TCCCGCTAAT 540
TTCATCTGTA TAGCCCAATA AGAAACTCTT GCCTGCTGTT AAAACAGGGG TATTAAAATT 600
GTCATCGTAC TCTGTAGATT TGACAATATA TTTTGTTGGT TGCTCATAGT TAAATACCTC 660
CCCCAACTTA CACTGCTCCC ATTCGTCACT AAATCCTTCA AACCGAATAG CTGGATACCC 720
GCTCTTATAA GCGAACATTT TCTGCAGTAA AGCGCTTTTT AAGCATTTAA GTTGCTGTTT 780
CTTTTCCTCA TGTAAAGTGA TTGCAGTATC CAATTCAGAG AAGAAGTTAG CAATTCTTTC 840
TTGTTCAGAC GTAGTTGGAA ACGCAACAGA CTGATTTCCG ACAATATCCC AGTTCAAATT 900
AACCTGACTT CCCGGCTGAC CATATTTGTT CCAATATGGT TTGAACATAA GAAGCCATTG 960
AAACATAAAT TCCTTATTAA ATGTTGGGTT GAGAAATATT AAGAATCCAT CGTGAACTCC 1020
TGTGTTAACG TAATTGATCA CTGGACTACC CACAGTAGCA GCAATACTTA ACAATAAATG 1080
TGGTTCTGTG ATAACACGCG TTTTAGATTG ACCAGCTTTT GAAATGTGTT GCGATAAGTG 1140
ATGAATGCGT CCTTTTTGTT CAGTGACATC GGATATTCTT AGCCATCCAA CATTTGAATT 1200
ATCATCGAAC CATTTGGGGT TAGAAATAGG TCTTGGACTC GCTCCACGTA CGATTTCCGC 1260
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TCAAATTAAA AATCAGCAAA ACCTATTTTG TGTCTGGTGG AACCAACAAG CGGCTAGAAA 1440

ATATGCTGCC AAACACCCTA AAGAACAAAA TATTGATAAC GAGCATACTT GGCATTAAAC 1500

GCCGTATAAG CTCATTTAAG CCGTTTTAAG TGTTATATGC ATAATTATAT TAAAACTGCT 1560

TTAAAATCGC TTAGAAGCAA GAATAGGCAG CTTGAGTGGC TGAATTGGCG ATGACTGAAC 1620

TAAGGACTAG GCCAAGAAAC TTTTGCACAG TCAACAATTC CCCGGACTAA TTCGGACTTT 1680

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TATAATAATA ATCAAGATTG ACAAGAGGAG GGCTGACAAT GGCAAATAGC GCTGGCATGC 1800

TGTCAGTAGG TCAAATAGCT AAAATGCTGA AGACCAACAG ACAGAACATT TACAACGTGC 1860

TTAAAGCTGA GCATATTAAA CCTGACGGCT TCAATGACAA GCACTATTCA CTTTACAGCC 1920

CGGAAACAAT TCAAGAGATC AAGGCCGCTC TGTCTAAGAA GGCAACGCTG AGAAGTAAGA 1980

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AGCTCAGGAG TCAGAACAGC CAGTTACAGC TAGACAATGC AAAGACTCAG CTCCTTATTG 2160

GCCAGGTTGA CCAGGAGAAG ACAACACTGA AGGCCGAGAA TGACCGACTG AGCGCTGAAA 2220

ATAACAAACT AGGACAATTA ACCGATAAGG TGCTGAAGGA CGCTCAGAGA GCAGAAGAGG 2280

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GGAATTGGGC GATAAGGAAG CCCGTCATTG CAAGGATAGA AGGTTAGTTC CAATAAGACA 2520

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GGAAAACTGT TATTTGCGTG TCAGCAAGAC AGAAGACGGC TATGACCTGG CTTTTGAACC 3000

ACAACAGCCT TCAAGAGAAT TGTTATGGTA CGATGATCTT TATTGGCGGC TGGGTAAAAC 3060

AGCTGACCAA CTCAAAACGT AAATCTAAGG TCAAGACGCT AGAGGAAATT ATCTAATGGC 3120

GGTTTACAAA GAATGGACTG ATTCAGATCA TTTAGAGTTA GTCAAAAATT GGAAATTACA 3180

CGGGCTGACT AACGTTGAGA TAGCTCAAAG AATAGGCATT GCTGAGAAGA CTTTGTACGT 3240

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GTAAACACAA GATAGCGGAA AATCTTCTGA TTATTATATT TACAAGCACT GTATATTGTG 3720

CTATTCTAAG ATGTGCTAAA CGGATTTGGG CAATGCAACT AACTGCTGTA AGGTATCAAC 3780

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CGGGTATTGC TTCACGGGTT CGCTCTTATT TTTTTATCTG GCTAGTTGCC TACTGGTACT 3900

ATGCTGACAC CCTAGCGGCA TGTTTGCGGT ATTGCACTAC AGCGGCAACA ATGGTAAAAA 3960

TAATAATAGG TAACAAAAAA GCCTTTAGTA CTGGCAATAC TAGAGGCGGG CTGTGTTTAG 4020

CTCTGGCAAA GCTTAACACG GTTAGAATTA TATTCCGTAC CACATATGAT ACGTTTAAAC 4080

GTAACACTCT GTCAAGGAGA ACATATCACC TTAAGGGTAC ATATAGTAGT TTTCTTCTAA 4140

CATTATGTTG TAAAAACATA ACATTTTGTA GACAAACACT ATACTTCTAT GACTCTAACC 4200

ATGTTTAAGA CAGGCCAGGC TAACACCTAT TGGCCTGTTT TTTGTTGCCA AAATTTCAAA 4260

AGAAAGGCGG TAACAGCCGT GATTAAACAA CAAAACATTG ATGTTAGAGC GGCTATTAAA 4320

GCTTCTGGTC TGAAGCAATA TGAGGTAGCT ACTTTGATGA ATGTTTCAGC TAGCTATCTC 4380

AGCCAGCTTT TACTTCAACC ATTGTCAGAA GGCCATAAGA AGCGCATTAT GGCGGCGATT 4440

AAACAAGGCG AGTCATTGAA GGGAGAACAA GAATAATGAT GAGCTTAGAA GAACGTGAGC 4500

AAGAAATTGA AAAGGTAGTA CGCATTGCTG AAGCTGACTT CAACAACGCT TGTCAATTGC 4560

ATGCTATCAA CAAGGAAGAT GTTATTAAGA ACCATGCTTA CAAGTATGCT GAAGTGCTGA 4620

GGCTTCAGGA ATTGCTGGCA TTGAACAAGA CCATTAGGGA CGGTCTGAAC GGCATTGAAA 4680

TGTCAGTAGA TCTCATTGAG TAGCGGGGAG ACCCGCCATG AACAACAGTG AAAAAAACTC 4740

TCTAATGGCT GAACCGTATA ACTCAGACCG CAACGCCATT GACAGACTCA GAATCAACCA 4800

GAAGGCCTTA CAGGCGGGCT CTGTCAAGCG TGAAGAGGGC TACAACTCAG AGGGCTTAGA 4860

AATGGTCTCC TACACGGCTT ATAAGAGCGG CATTCAGTAT GTCATTTCTT CAGAAGCTGA 4920

AGGCGGCAAA ATGGTTATTA ACGAGACCTT CAGCAAGGTT CAACATCTAC TAATTGCCAG 4980

CTGGTATAGC CAGCCAGACA GAGCCAGCAA TTTCAGAATA CAGCTGACCT TTAAAGAGAT 5040

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CTTCAGCCTA AACTGTATGT TTTAGGCGCT GTCAGTTCGC TAGGGAAAAC GACTTTTGCC 7140

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GACAATAGAG TTAAGGCAAG TCAGGTAAAA GACCTGGTCA ATAGTTGGCT TGACAACCAC 7440

CCGGACGAGA AGAAGCCGCT TGTAGTCGTT GACTATCTTC AGATCTTGCA AGCTGAGCAG 7500

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TAGACCCGGT AACAGGCCTG GCAACAGAGA AGAAGCCCGA TAAATAGAAC TGAAGAAGCT 8040

GGCCAGGAAT GGCTGGCTTT TGTTTTGCCT TCAGACGCTC TCAGAAGCTC ATAGAGCCCC 8100

TCTGAGCCTG CATTGGTAGA TTTTTCCGGC CGAACACCCC 8140

(3) Information for sequence ID No 2.

(i) Sequence characteristics:

(A) Length: 1202 base pairs
(B) Type: Nucleic acid
(C) Strandedness: Double
(D) Topology: Linear

(ii) Molecule type: DNA (synthetic)

(xi) Feature:
(vi) Origional source: Lactobacillus bulgaricus
(A) Name/key: lacS promotor
(B) Location: 1..239

(ix) Feature:
(vi) Origional source: Staphylococcus aureus
(A) Name/key: Chloramphenicol acetyltransferase peptide
(B) Location: 240..890

(ix) Feature:
(vi) Origional source: Lactococcus lactis
(A) Name/key: stem-loop terminator following galT gene
(B) Location: 903..1102

GAATTCACCA ACGCTTTCAT TTCACGCCTC CCGAAGTACA TGCAAGAGGC TATATCGCCA 60

TCATTAGCAG CTTAATTGAA TATTTACTGG CTAAACTATT GAGTTTTCAA GGCTTCATAG 120

TTCTTTTTGG TGTGGAAGTT TAAATTACTA AAAATATTTT AGTAAAACAT CTTGGTTTAT 180

TTAGTAAACA AGTCTATACT GTAATTATAA ACAAGTTAAC ACACCTAAAG GAGAATTTCA 240

TGAACTTTAA TAAAATTGAT TTAGACAATT GGAAGAGAAA AGAGATATTT AATCATTATT 300

TGAACCAACA AACGACTTTT AGTATAACCA CAGAAATTGA TATTAGTGTT TTATACCGAA 360

ACATAAAACA AGAAGGATAT AAATTTTACC CTGCATTTAT TTTCTTAGTG ACAAGGGTGA 420

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GGACTCCTGT AAAGAATGAC TTCAAAGAGT TTTATGATTT ATACCTTTCT GATGTAGAGA 600

AATATAATGG TTCGGGGAAA TTGTTTCCCA AAACACCTAT ACCTGAAAAT GCTTTTTCTC 660

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TACCGCTATC TTTACAGGTA CATCATTCTG TTTGTGATGG TTATCATGCA GGATTGTTTA 840

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AAGACAGTTA AGAAGAAATA AAAATAAATT TAAAAGAGTA TCACTAGCTT TTTTTGGTTT 1080

AGTGATTATT TTAGCGGAGC TC 1102

SEQUENCES LISTING

(1) GENERAL INFORMATION :

(i) APPLICANT:
(A) NAME: SOCIETE DES PRODUITS NESTLE S.A.
(B) STREET ADRESS: P.O.Box 353
(C) CITY: VEVEY
(E) COUNTRY: SWITZERLAND
(F) POSTAL CODE: 1800
(G) TELEPHONE: (21) 924 21 39
(H) FAX: (21) 921 18 85
(I) TELEX: 451 311

(ii) TITLE OF INVENTION: Plasmid derived from Lactobicillus bulgaricus

(iii) NUMBER OF SEQUENCES: 6

(iv) MANDATORY INFORMATIONS:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) Information for SEQ ID NO: 1:

(i) Sequence characteristics:

(A) Length: 8140 base pairs
(B) Type: Nucleic acid
(C) Strandedness: Double
(D) Topology: Circular

(ii) Molecule type: DNA (plasmid)

(vi) Origional source: Lactobacillus bulgaricus Strain N2.
(A) Name/key: Plasmid pN42
(B) Location: 1..8140

(ix) feature:
(A) Name/Key: Origin of replication.
(B) Location: 5694..5758.

(ix) feature:
(A) Name/Key: ORF1.
(B) Location: 1344..169.

(ix) feature:
(A) Name/Key: ORF2.
(B) Location: 5965..7806.

(ix) feature:
(A) Name/Key: ORF3.
(B) Location: 4718..5668.

(ix) feature:
(A) Name/Key: ORF4.
(B) Location: 3116..3637.

(ix) feature:
(A) Name/Key: ORF5.
(B) Location: 1779..2360.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCTAGGCTTG AAATTGACGC ATAGGCGCAA AGGGAGCGGG CGACAGGGGG TAAAGCACGA 60

TAAATTCGTT TTTTACAGAC GTTCAGTCCA TGTTGTCATA TTTGTACTCC CGTTTTTAGG 120

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TTGTAAAAAG CCTTTTTTCT GTTCTTTCAA CAAATCTAAC TTACGTTGAT GAAGAGCGAT 240

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GGCCAGGAAT GGCTGGCTTT TGTTTTGCCT TCAGACGCTC TCAGAAGCTC ATAGAGCCCC 8100

TCTGAGCCTG CATTGGTAGA TTTTTCCGGC CGAACACCCC 8140

(3) Information for SEQ ID NO: 2:

(i) Sequence characteristics:

(A) Length: 1202 base pairs
(B) Type: Nucleic acid
(C) Strandedness: Double
(D) Topology: Linear

(ii) Molecule type: DNA (synthetic)

(vi) Origional source: Lactobacillus bulgaricus
(A) Name/key: lacS promotor
(B) Location: 1..239

(vi) Origional source: Staphylococcus aureus
(A) Name/key: Chloramphenicol acetyltransferase peptide
(B) Location: 240..890

(vi) Origional source: Lactococcus lactis
(A) Name/key: stem-loop terminator following galT gene
(B) Location: 903..1102

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAATTCACCA ACGCTTTCAT TTCACGCCTC CCGAAGTACA TGCAAGAGGC TATATCGCCA 60

TCATTAGCAG CTTAATTGAA TATTTACTGG CTAAACTATT GAGTTTTCAA GGCTTCATAG 120

TTCTTTCTGG TGTGGAAGTT TAAATTACTA AAAATATTTT AGTAAAACAT CTTGGTTTAT 180

TTAGTAAACA AGTCTATACT GTAATTATAA ACAAGTTAAC ACACCTAAAG GAGAATTTCA 240

TGAACTTTAA TAAAATTGAT TTAGACAATT GGAAGAGAAA AGAGATATTT AATCATTATT 300

TGAACCAACA AACGACTTTT AGTATAACCA CAGAAATTGA TATTAGTGTT TTATACCGAA 360

ACATAAAACA AGAAGGATAT AAATTTTACC CTGCATTTAT TTTCTTAGTG ACAAGGGTGA 420

TAAACTCAAA TACAGCTTTT AGAACTGGTT ACAATAGCGA CGGAGAGTTA GGTTATTGGG 480

ATAAGTTAGA GCCACTTTAT ACAATTTTTG ATGGTGTATC TAAAACATTC TCTGGTATTT 540

GGACTCCTGT AAAGAATGAC TTCAAAGAGT TTTATGATTT ATACCTTTCT GATGTAGAGA 600

AATATAATGG TTCGGGGAAA TTGTTTCCCA AAACACCTAT ACCTGAAAAT GCTTTTTCTC 660

TTTCTATTAT TCCATGGACT TCATTTACTG GGTTTAACTT AAATATCAAT AATAATAGTA 720

ATTACCTTCT ACCCATTATT ACAGCAGGAA AATTCATTAA TAAAGGTAAT TCAATATATT 780

TACCGCTATC TTTACAGGTA CATCATTCTG TTTGTGATGG TTATCATGCA GGATTGTTTA 840

TGAACTCTAT TCAGGAATTG TCAGATAGGC CTAATGACTG GCTTTTATAA TATGAGATAA 900

TCGAAAAAAA AAAGCTCAAA TTTTTGAGCT TTTTTTGTAT GTAATTGTCA TGCATGAAAA 960

TGTAATGGTA ATTGTGATAA TTATTAATAA AAAAATTGAT ATAATGAAGT GGATGAAAAA 1020

AAGACAGTTA AGAAGAAATA AAAATAAATT TAAAAGAGTA TCACTAGCTT TTTTTGGTTT 1080

AGTGATTATT TTAGCGGAGC TC 1102

(4) Information for SEQ ID NO: 3:

(i) Sequence characteristics:

(A) Length: 33 base pairs
(B) Type: Nucleic acid
(C) Strandedness: Single
(D) Topology: Linear

(ii) Molecule type: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AGGAGGATCC TCTCATGAAC TTTAATAAAA TTG 33

(5) Information for SEQ ID NO: 4:

(i) Sequence characteristics:

(A) Length: 26 base pairs
(B) Type: Nucleic acid
(C) Strandedness: Single
(D) Topology: Linear

(ii) Molecule type: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TACAGTATCG ATTATCTCAT ATTATA 26

(6) Information for SEQ ID NO: 5:

(i) Sequence characteristics:

(A) Length: 31 base pairs
(B) Type: Nucleic acid
(C) Strandedness: Single
(D) Topology: Linear

(ii) Molecule type: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ATTGGAAGAA TTCACCAACG CTTTTCATTT C 31

(7) Information for SEQ ID NO: 6:

(i) Sequence characteristics:

(A) Length: 19 base pairs
(B) Type: Nucleic acid
(C) Strandedness: Single
(D) Topology: Linear

(ii) Molecule type: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GGTGGTGACG AAGACGATA 19

## Claims

1. Plasmid derived from Lactobacillus delbrueckii sp. comprising at least the restriction map of the Figure 1 or portion(s) thereof.

2. Plasmid according to claim 1, characterized in that the portion is a sufficient amount of the restriction map of the Figure 1, so as to provide all the plasmid encoded TRANS and CIS elements necessary for replication of the plasmid in Lactobacillus bulgaricus.

3. Plasmid according to claim 1 or 2 comprising at least the DNA sequence SEQ ID N° 1 and/or its complementary strand or portion(s) thereof.

4. Plasmid according to claim 3, characterized in that the portion is a sufficient amount of the DNA sequence SEQ ID N° 1, and/or its complementary strand, so as to provide all the plasmid encoded TRANS and CIS elements necessary for replication of the plasmid in Lactobacillus bulgaricus.

5. Recombinant vector comprising the plasmid according to any of the preceding claims, at least one DNA sequence capable of replication in E. coli and/or Lc. lactis and at least one marker.

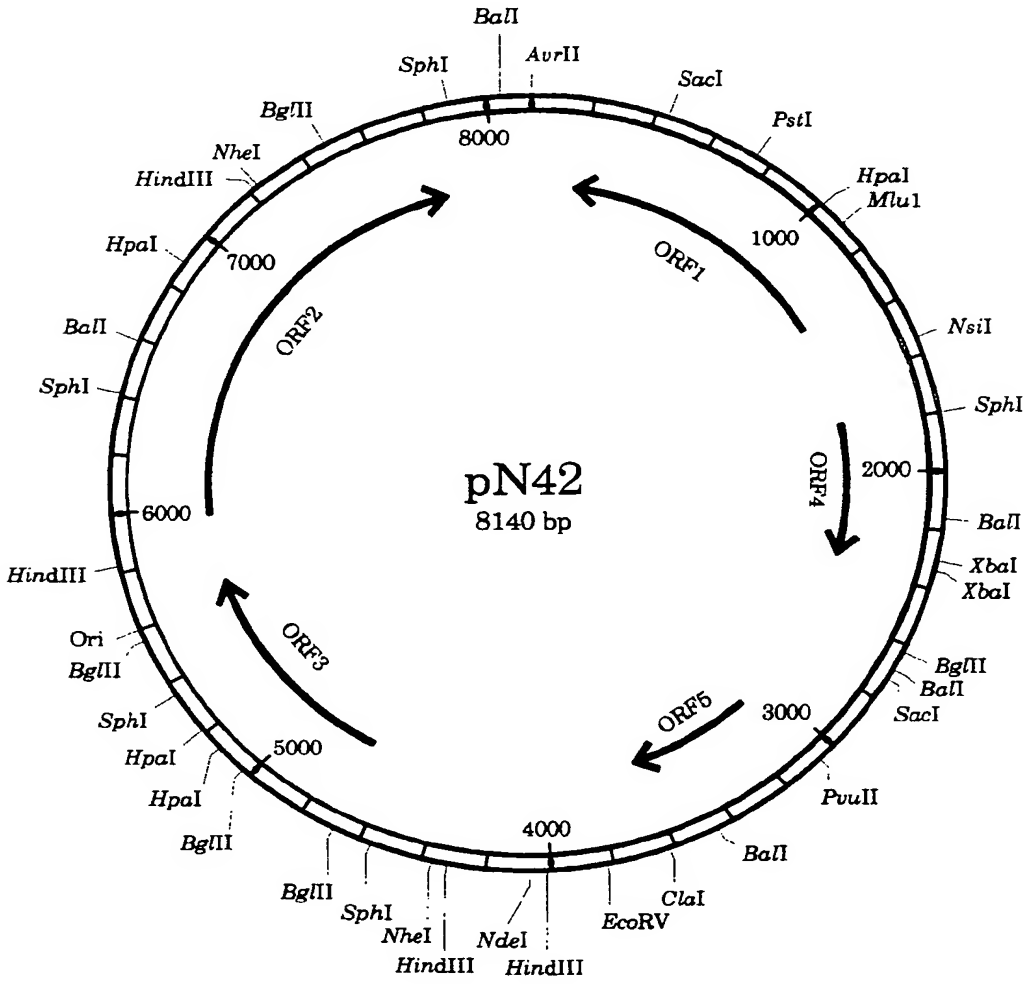
6. Microorganism transformed by the plasmid according to any of the claims 1 to 4 and/or by the recombinant vector according to claim 5.

7. Lactobacillus bulgaricus transformed by the plasmid according to any of the claims 1 to 4 and/or by the

recombinant vector according to claim 5.

8. Use of the plasmid according to any of the claims 1 to 4 and/or the vector according to claim 5 for the transformation of microorganisms.

FIG.1



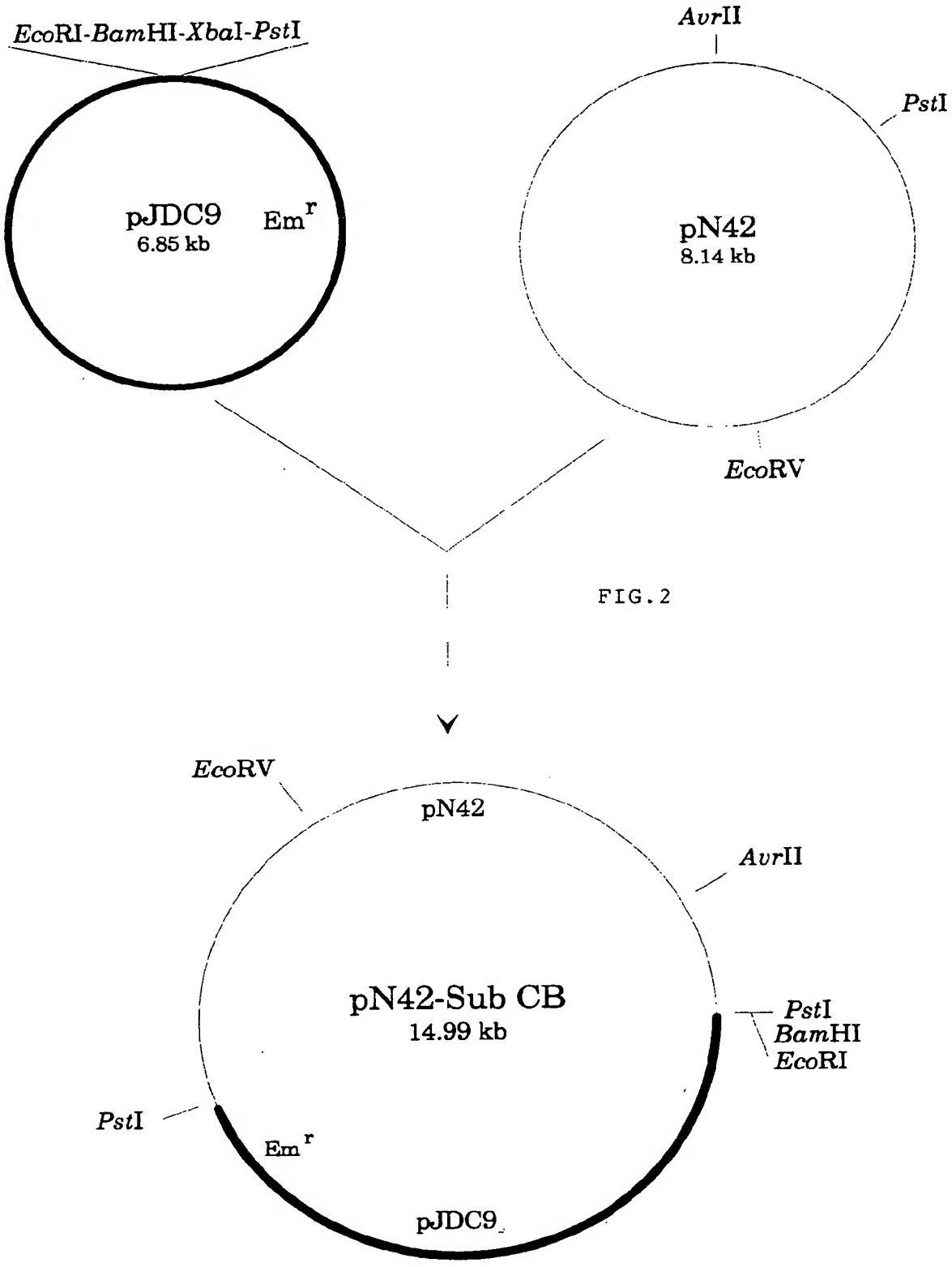



FIG.2

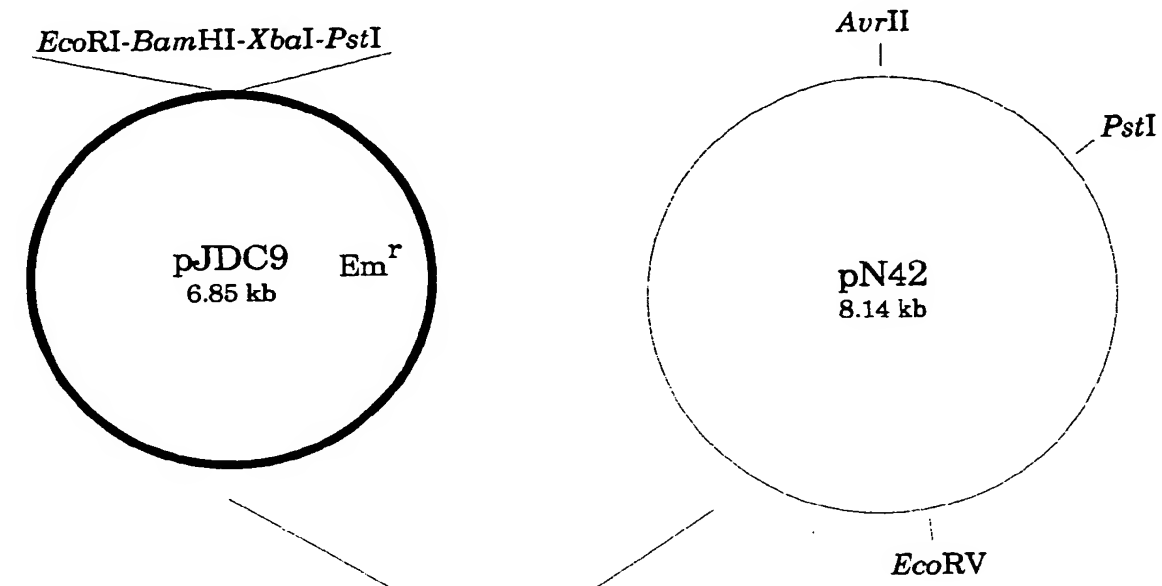
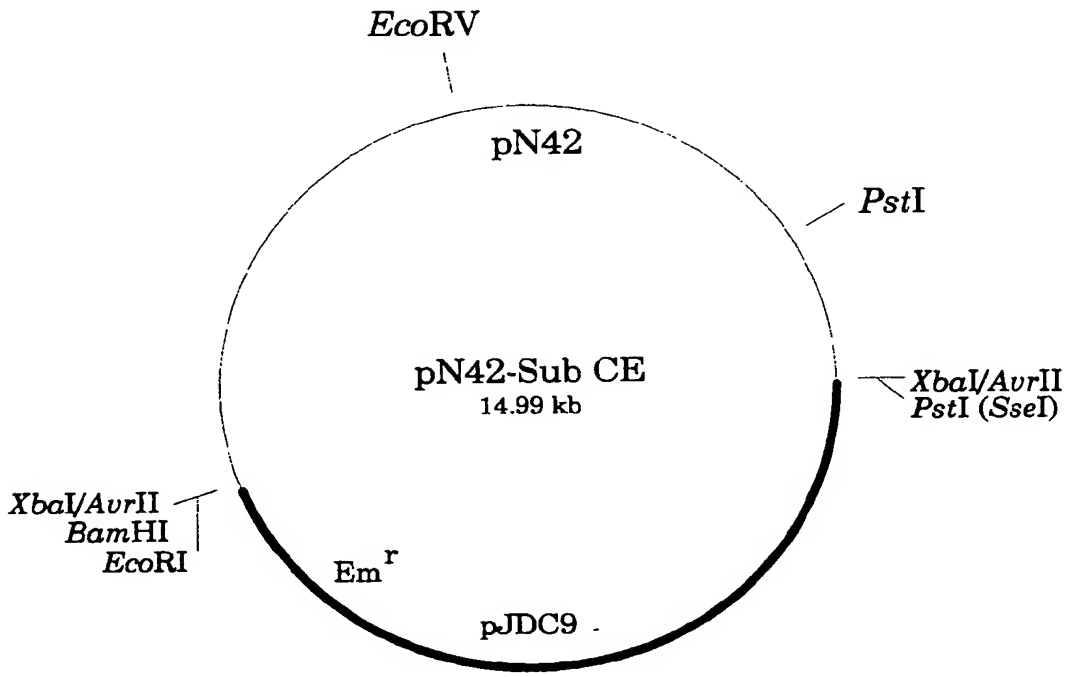



FIG. 3



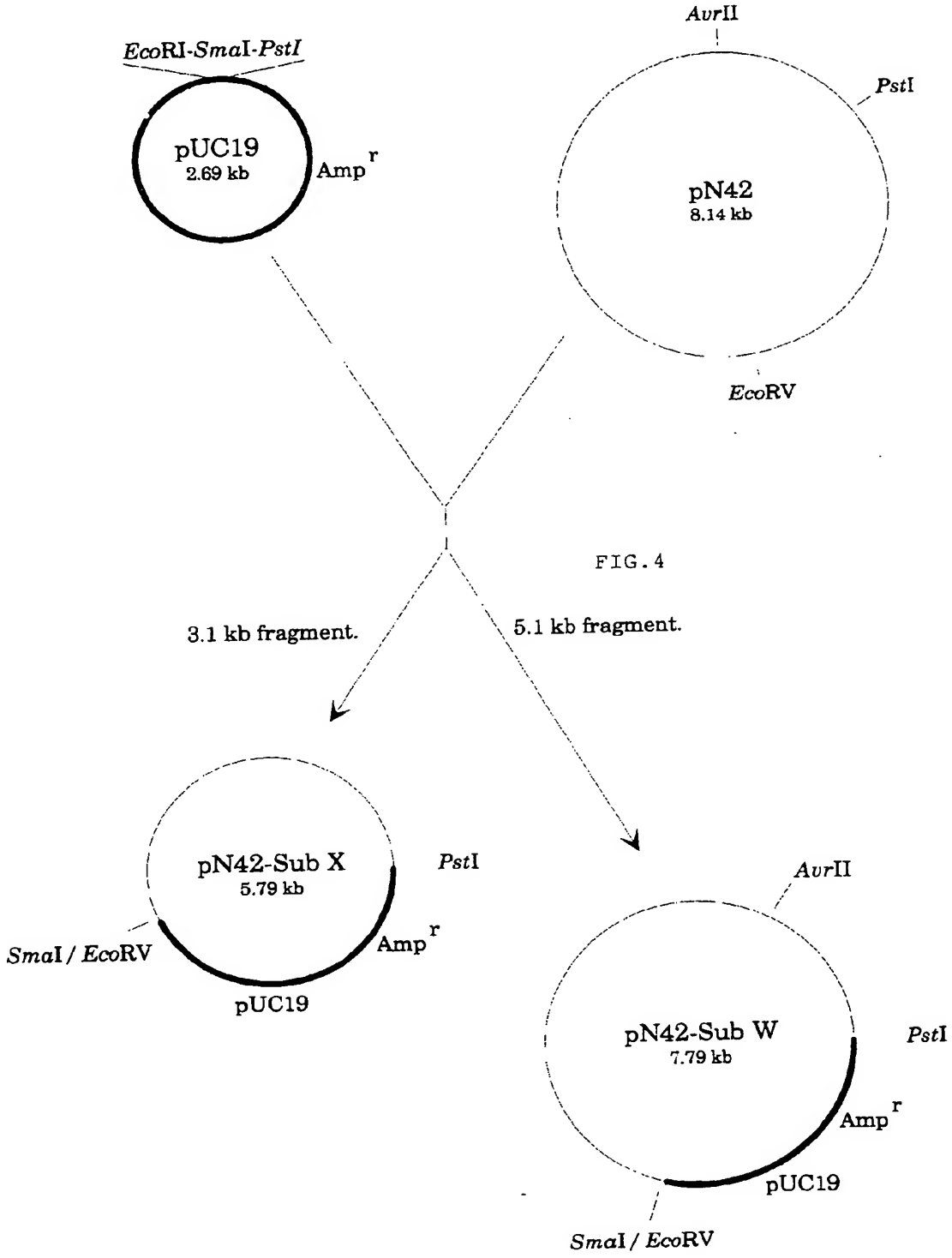



FIG. 4

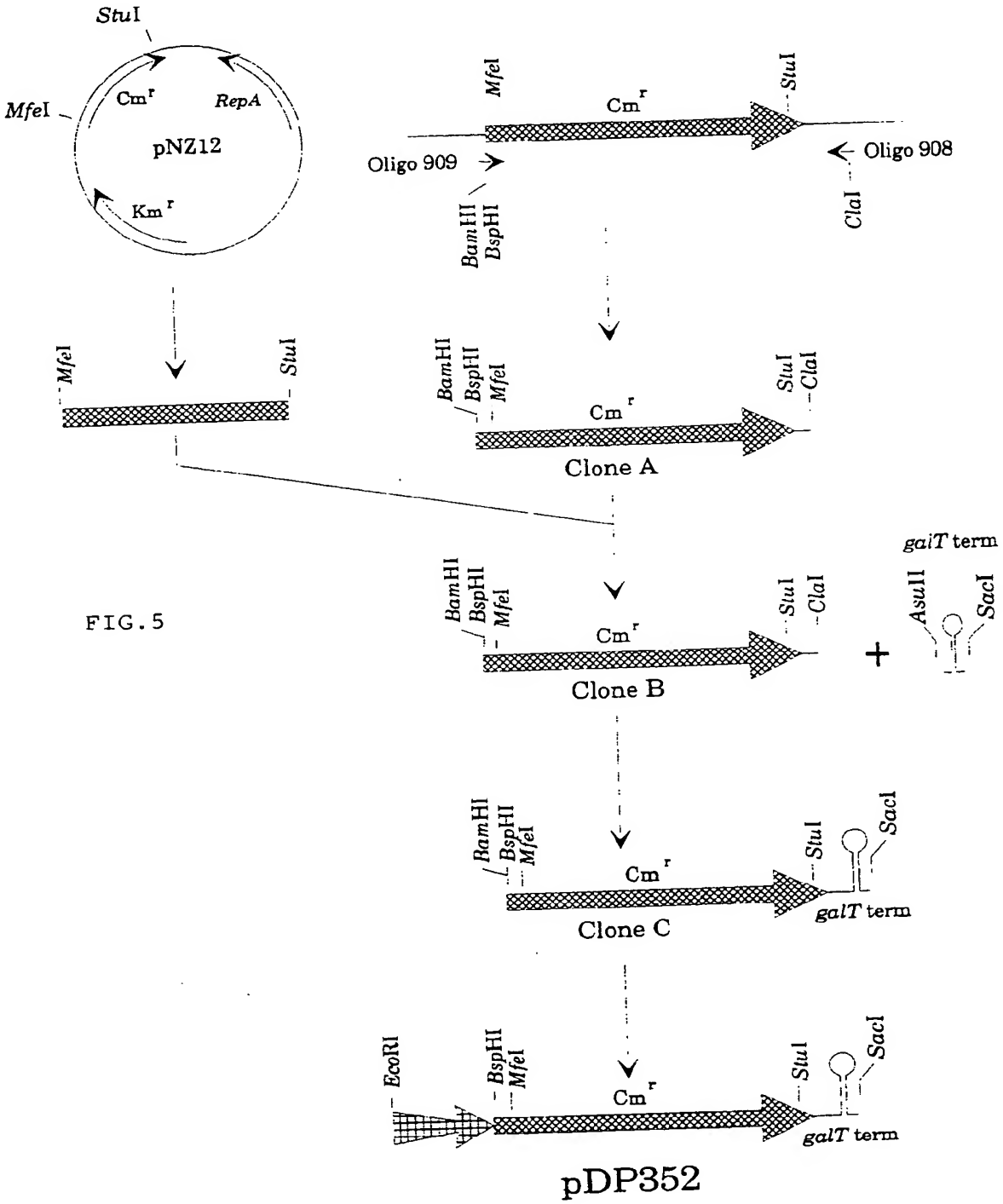



FIG.5

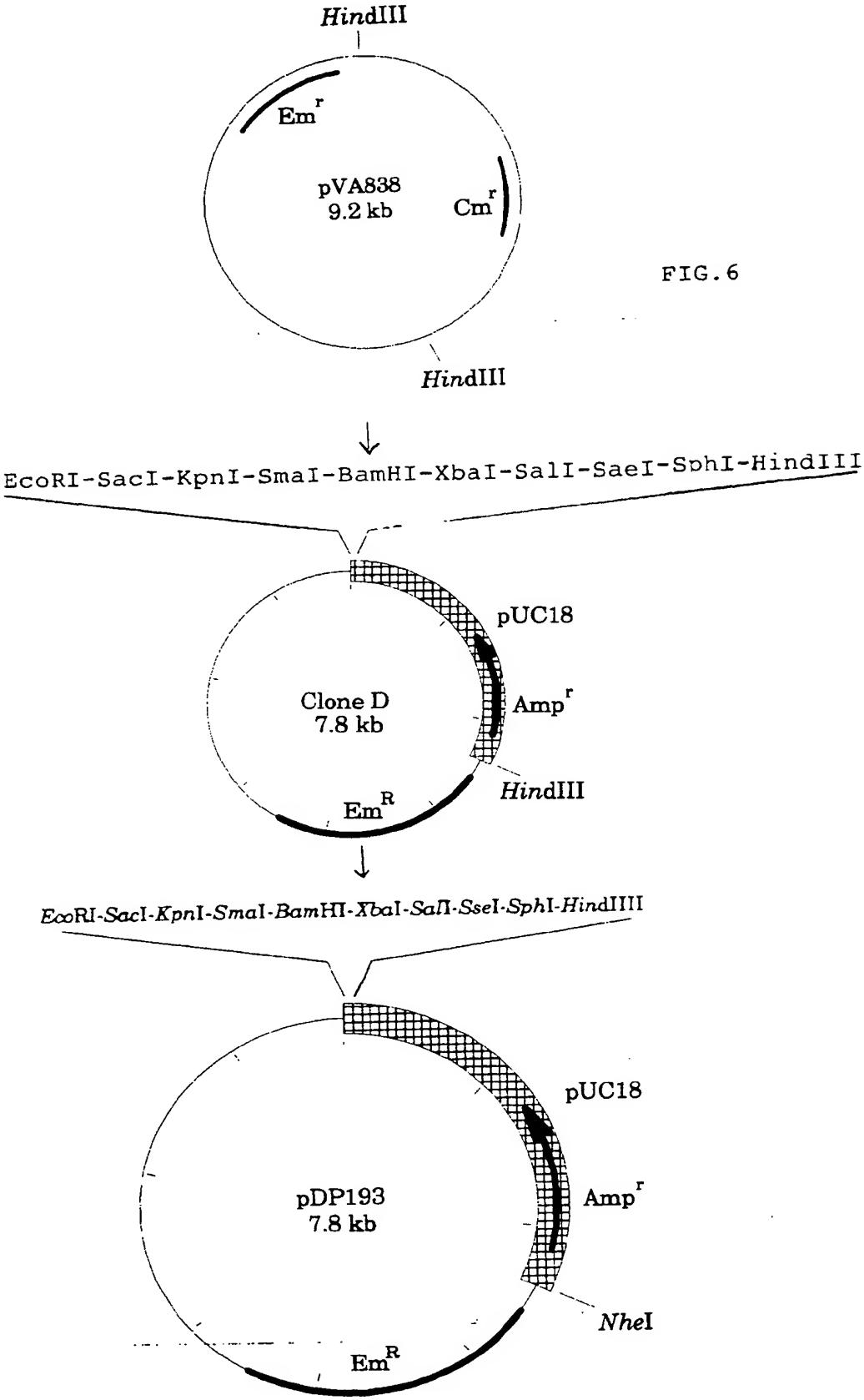



FIG.6

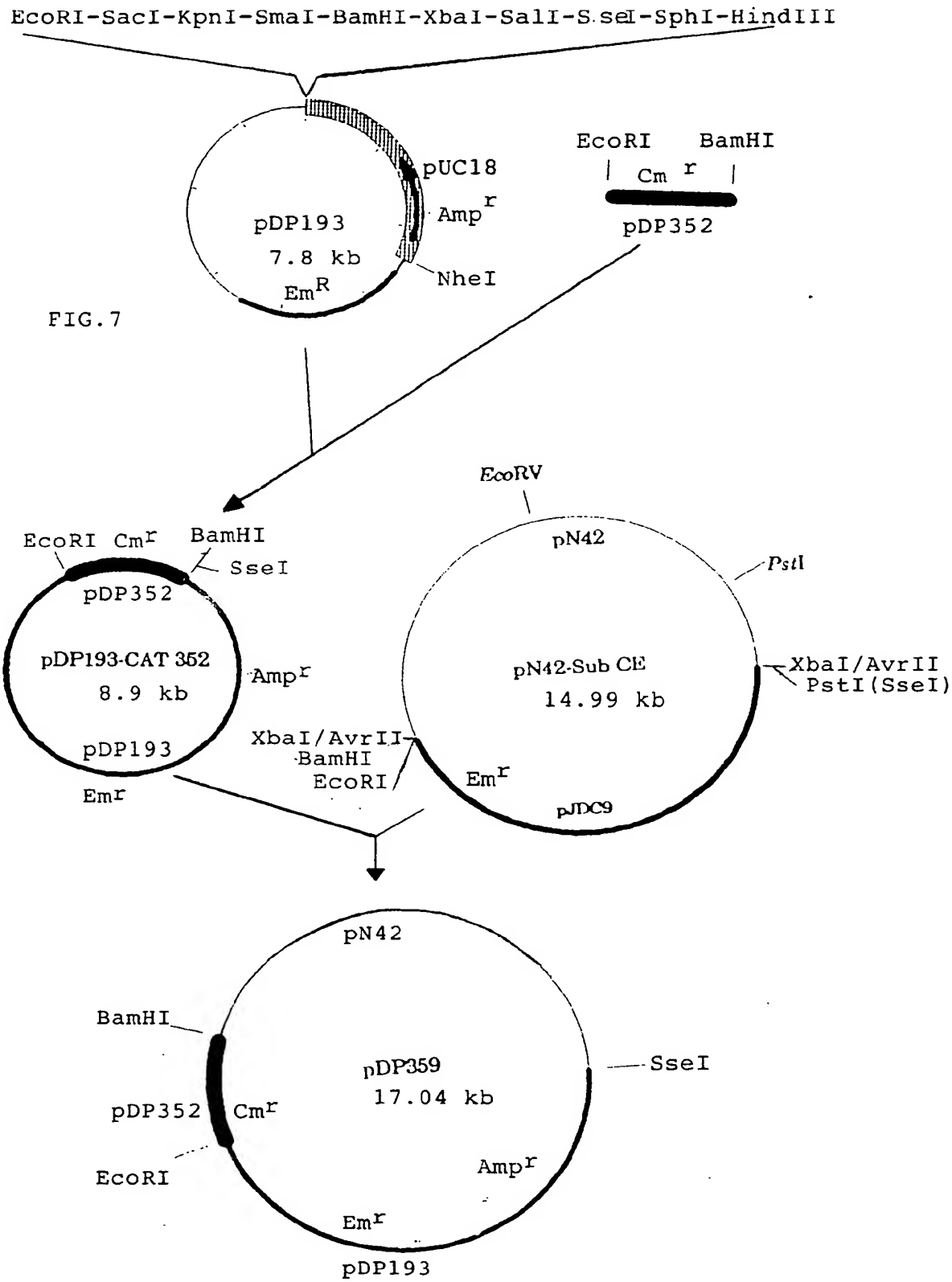



FIG.7

European Patent Office

# EUROPEAN SEARCH REPORT

Application Number
EP 94 20 2468

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int.Cl.6) |
|---|---|---|---|
| D,A | EP-A-0 529 088 (MEIJI MILK PROD. CO., LTD.) 3 March 1993<br>* the whole document *<br>--- | 1-8 | C12N15/74<br>C12N1/21<br>//(C12N1/21, C12R1:225) |
| A | JAPANESE PATENTS ABSTRACTS (UNEXAMINED)<br>Week 9238,<br>Derwent Publications Ltd., London, GB;<br>AN 92-312519<br>& JP-A-4 218 381 (SNOW BRAND MILK PROD CO LTD) 7 August 1992<br>* abstract *<br>--- | 1-8 | |
| A | CAN. JOURNAL OF MICROBIOLOGY,<br>vol.38, 1992, NATL. RESEARCH COUNCIL,OTTAWA,CAN;<br>pages 69 - 74<br>P. CHAGNAUD ET AL. 'Construction of a new shuttle vector for Lactobacillus'<br>* the whole document *<br>--- | 1-8 | |
| A | ACTA MICROBIOLOGICA BULGARICA,<br>vol.27, no.0, 1991, BULGARIAN ACADEMY OD SCIENCES,SOFIA, BULGARIAN;<br>pages 3 - 8<br>V. MITEVA ET AL. 'Isolation and characterization of plasmids from different strains of Lactobacillus bulgaricus, Lactobacillus helveticus and Streptococcus thermophilus'<br>* the whole document *<br>--- | 1-8 | TECHNICAL FIELDS SEARCHED (Int.Cl.6)<br>C12N |
| D,A | APPLIED AND ENVIRONMENTAL MICROBIOLOGY,<br>vol.56, no.6, June 1990,<br>AM.SOC.MICROBIOL.,WASHINGTON,DC,US;<br>pages 1967 - 1970<br>M. DELLEY ET AL. 'DNA probe for Lactobacillus delbrueckii'<br>* the whole document *<br>--- | 1-8 | |
| | -/-- | | |

The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| THE HAGUE | 16 December 1994 | Hornig, H |

CATEGORY OF CITED DOCUMENTS

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Application Number
EP 94 20 2468

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int.Cl.6) |
|---|---|---|---|
| D,A | JOURNAL OF BACTERIOLOGY, vol.173, no.6, March 1991, AM. SOC. MICROBIOL., BALTIMORE, US; pages 1951 - 1957<br>P. LEONG-MORGENTHALER ET AL. 'Lactose metabolism in Lactobacillus bulgaricus: Analysis of the primary structure and expression of the genes involved'<br>* the whole document *<br>----- | 1-8 | |

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

The present search report has been drawn up for all claims

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|---|---|---|
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